Macrophage Migration Inhibitory Factor Promotes Colorectal Cancer

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A growing body of evidence implicates macrophage migration inhibitory factor (MIF) in tumorigenesis and metastasis. In this study, we investigated whether MIF expression was associated with clinicopathologic features of colorectal carcinoma (CRC), especially in tumors with hepatic metastasis, and whether neutralization of endogenous MIF using anti-MIF therapeutics would inhibit tumor growth and/or decrease the frequency of colorectal hepatic metastases in a mouse colon carcinoma model. The concentration of serum MIF was positively correlated with an increased risk of hepatic metastasis in human patients with CRC ($R = 1.25$, 95% confidence internal = 1.02–1.52, $P = 0.03$). MIF was also dramatically upregulated in human colorectal tissue, with 20–40 times as many MIF-positive cells found in the mucosa of patients with CRC than in normal tissue ($P < 0.001$ ANOVA). Moreover, in those patients with metastatic colorectal cancer in the liver, MIF-positive cells were similarly increased in the diseased hepatic tissue. This increased MIF expression was restricted to diseased tissue and not found in areas of the liver with normal morphology. In subsequent *in vitro* experiments, we found that addition of recombinant MIF to colonic cell lines significantly increased their invasive properties and the expression of several genes (for example, matrix metalloproteinase 9 and vascular endothelial growth factor) known to be upregulated in cancerous tissue. Finally, we treated mice that had been given CT26 colon carcinoma cell transplants with anti-MIF therapeutics—either the MIF-specific inhibitor ISO-1 or neutralizing anti-MIF antibodies—and observed a significant reduction in tumor burden relative to vehicle-treated animals. Taken together, these data demonstrate that MIF expression was not only correlated with the presence of colorectal cancer but also may play a direct role in cancer development.

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INTRODUCTION

Despite advances in diagnosis and treatment, colorectal cancer remains a major cause of cancer death worldwide. It is the second most common malignancy and the second most common cause of cancer death in Europe (1) and the United States (2). Liver metastasis associated with colorectal cancer is an important risk factor; approximately half of all patients with colorectal cancer develop liver metastasis and die within 5 years after diagnosis. Elucidation of the molecular mechanisms underlying the invasive and metastatic properties of colorectal cancer is thus of great interest.

Macrophage migration inhibitory factor (MIF), originally identified as a product of activated lymphocytes, has been found to have multiple functions including catalytic activity, lymphocyte immunity, endocrine regulation, signal modulation, and proinflammatory action (3). In addition to the pivotal effects of MIF on the immune system and inflammatory response, several reports have linked MIF to fundamental processes that control cell proliferation, differentiation, angiogenesis, tumor progression, and metastasis (4–11). For example, Hudson and coworkers have shown that MIF can abolish the tumor suppressive activity of p53 (11). Recently, MIF overexpression has been reported in metastatic prostate cancer (12), breast carcinoma (13), hepatocellular carcinoma (14), and lung adenocarcinoma (15). MIF has also been implicated in melanoma tumor growth and angiogenesis, and treatment with anti-MIF antibodies inhibited tumor angiogenesis in a human melanoma model (9). Similarly, increased MIF expression is associated with both enhanced proliferation of

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murine colon cancer cells in response to growth factors (6) and with loss of cell differentiation and lymph node metastases (16). Li and coworkers demonstrated that MIF secreted by infiltrating lymphocytes may induce matrix metalloproteinase 9 (MMP-9) and interleukin-8, and thus contribute to the invasion and metastasis of nasopharyngeal carcinoma cells in the early stages (17). Tumor-derived MIF, acting as an autocrine factor, enhanced the production of vascular endothelial growth factor (VEGF) and interleukin-8, and hence promoted angiogenesis and tumor growth in esophageal cancer (16).

The development of colorectal cancer is a multistep process from normal mucosa to colorectal adenoma (a precancerous lesion), and finally to invasive cancer. In this study, we investigated the expression of MIF in normal colorectal mucosa and colorectal adenoma and carcinoma, and assessed the role of MIF in the carcinogenesis and metastasis of colorectal cancer. We demonstrated that MIF abundance is upregulated in both the serum and colorectal tissue of patients with colorectal cancer relative to that seen in disease-free individuals. Consistent with this correlation, we show that the addition of recombinant human MIF (rMIF) increases the invasive properties and expression of cancerous genes *in vitro*. Conversely, we demonstrate that inhibition of endogenous MIF significantly reduces tumor burden in a mouse model of colorectal cancer.

MATERIALS AND METHODS

Determination of Serum Protein Levels

Human serum levels of MIF were measured in triplicate by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instruction (DMP900; R&D Systems, Minneapolis, MN, USA). MIF absorbance was read using a microtiter plate reader (NovaPath; Bio-Rad, Hercules, CA, USA) at 450 nm. The sensitivity of the MIF assay was 0.017 ng/mL. Mouse MIF and MMP-9 serum levels were also measured in triplicate by ELISA, according to the manufacturer's instruction (Shen Xiong. Shanghai, China).

Patients and Specimens

The paraffin embedded tissues used for immunohistochemistry were obtained from the Department of Pathology of the Second Affiliated Hospital of Guangzhou Medical College. A total of 77 patients (who underwent colorectal cancer diagnosis during the period 2003–2005) participated in this study, 8 with normal intestinal mucosa, 20 with colorectal adenomas, and 49 with colorectal carcinomas. In some cases, multiple samples were analyzed from patients by isolating tissues from different regions (for example, 16 relatively normal intestinal mucosa from noncancerous regions at least 5 cm away from the primary tumor, 10 carcinomas from patients with hepatic metastasis, and 5 relatively normal hepatic tissues from noncancerous regions at least 5 cm away from the primary tumor in patients with hepatic metastasis). All patients underwent surgical resection or biopsy and did not receive neoadjuvant treatment. All specimens were pathologically confirmed. The postsurgical pathologic TNM (tumor, nodes, and metastases) stage was determined according to the guidelines of the American Joint Committee on Cancer. Blood samples were collected from 20 healthy volunteers, 12 colorectal adenoma patients, and 30 colorectal carcinoma patients, before surgical resection and endoscopy, corresponding to the specimens measured by immunohistochemistry. The sera aliquots were stored at –80°C until used in ELISA assay. This project was approved by the Ethics Committee of Guangzhou Medical College in China.

Immunohistochemistry of MIF in colorectal and hepatic tissues. Formalin-fixed, paraffin-embedded tissues were immunohistochemically stained for MIF. In brief, deparaffinized and rehydrated sections (2 μm) were pretreated with 3% H_2O_2 for 2 min at room temperature to block endogenous peroxidase activity, and were processed for antigen retrieval in citrate buffer, pH 6.0 (Dako, Carpinteria, CA, USA), for 15 min in a microwave oven. Sections were then washed three times with phosphate buffered saline (PBS), pH 7.4, and incubated with a rabbit antihuman MIF polyclonal antibody (FL-115, 1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 75 min at 37°C. After three additional washings with PBS, sections were incubated with EnVision^{TM} (Dako) for 45 min at 37°C and were developed for 15 min with 3,3-diaminobezidine (DAB; Dako) to produce a brown color, and were then counterstained with hematoxylin. We used lung tumor sections as a positive control, and a negative control sample was prepared with the same procedure, except that PBS was substituted for anti-MIF antibodies. All samples were scored in a blinded fashion.

The cytoplasmic staining of MIF was quantitated by counting the number of MIF-positive cells in a total of 500 tumor cells in at least five randomly selected fields at high power (400×) under a standard light microscope. Because the colorectal carcinoma tissues contained too many positive MIF-staining cells, we used another assessment according to the extent of positive MIF staining, with pale yellow indicating weak-positive staining (1+); brown yellow, moderate-positive staining (2+); nut-brown yellow, strong-positive staining (3+).

Cell culture. Both the human colorectal carcinoma (CRC), cell line (LoVo), and mouse undifferentiated colon carcinoma cell line (CT26) were obtained from American Type Culture Collection (Manassas, VA, USA). These cells were cultured in RPMI1640 (Gibco-Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO, USA), and incubated at 37°C in a humidified 5% CO_2 incubator.

Cell invasion assay. Invasion activity was assayed in a transwell plate (Corning, Corning, NY, USA) using an 8-μm porosity polycaronate filter membrane. The upper layer of membrane inserts were precoated with 40 μg Matrigel per well (Becton Dickinson, Franklin Lakes, NJ, USA), a material that mimics the basement membrane. LoVo cells (100 μL, 1×10^5 cells/mL RPMI1640 with 1% FBS) with or without rMIF (250 ng/mL) were placed into the upper compartment of the transwell. The lower compartment of each well was filled with 600 μL of medium (RPMI1640 with 1% FBS). After a 24-h incubation at 37°C, noninvasive cells were removed from the upper chamber with cotton swabs, and the cells that migrated through the coated membrane to the lower compartment were stained with 1% crystal violet and quantitated. All invasion assays were done in triplicate.

Reverse transcription polymerase chain reaction (RT-PCR). We used Trizol reagent (Sangon, Shanghai, China) to isolate total RNA from the LoVo cells treated with or without rMIF (250 ng/mL) for 24 h. We performed semiquantitative RT-PCR in triplicate using a Qiagen Onestep RT-PCR Kit (Qiagen, Valencia, CA, USA). The PCR primers—sense, antisense, and (product size)—were as follows: MMP-2, 5′-GGCACCCATTTACACCTACACCAA-3′, 5′-GCTTCCAAACTTCACGC-TCTTCAG-3′ (694 bp); MMP-9, 5′-GACTCGGTCT TTGAGGAGCC-3′, 5′-GAACTCACGC GCCAGTAGAA-3′ (350 bp); β-actin, 5′-TCGACAACGGCTCCGGCAT-3′, 5′-GAAGGTGTGGTGCCAGATTTTCT-3′ (242 bp); VEGF, 5′-TCGGGCCTCC GAAACCATGA-3′, 5′-CCTGGTGAGA GATCTGGTTC-3′ (720 bp). All primers were manufactured at Sangong Biocompany, Shanghai, China. The thermal cycles were 50°C, 30 min for reverse transcription and 95°C, 15 min for initial PCR activation, followed by denaturing at 95°C for 45 s, annealing at 60°C for 45 s, extension at 72°C for 90 s, and final extension at 72°C for 10 min (MMP-2 and MMP-9) and 34 cycles for denaturing at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min (VEGF). The PCR products were analyzed on 1.5% agarose gel, stained with ethidium bromide, and analyzed by densitometry. Data were quantitated by normalizing to the internally amplified actin product using a ratio, which was defined as the production band area intensity divided by the β-actin band area intensity.

Tautomerase activity. To assess the biological activity of MIF, we used a modified tautomerase assay (18) on either CT26 cell lysates or mouse serum following treatment with the MIF-specific inhibitor (S,R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1) (19,20). In one assay, CT26 cells (1×10^6) were treated with ISO-1 (10 nM–100 μM) at 37°C for 30 min. The medium was then replaced with ISO-1–free medium, and the cells were washed repeatedly and lysed with 600 μL of ice-cold lysis buffer (Tris nondenaturing buffer: 20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM Na_2EDTA; 1 mM EGTA; 1% Triton; 2.5 mM sodium pyrophosphate; 1 mM β-glycerophosphate; 1 mM Na_3VO_4; 1 μg/mL leupeptin) with gentle rotation at 4°C for 20 min. In another assay, the serum of mice treated with saline, 5% dimethyl sulfoxide (DMSO), or ISO-1 (20 mg/kg in 5% DMSO) were collected following 4 wks of treatment (see below). Both assays were prepared with L-dopachrome methyl ester as follows: a fresh stock solution of L-dopachrome methyl ester was prepared at 2.4 mM through oxidation of L-3,4-dihydroxyphenylalanine methyl ester with sodium periodate. Tautomerase activities were determined at room temperature by adding dopachrome methyl ester (0.3 mL) to a cuvette containing 0.7 mL of the supernatants, and the spectrometric measurements were made at 475 nm for 20 s by monitoring the rate of decolorization of L-dopachrome methyl ester compared with the rate in a standard solution.

Animals

BALB/c male mice weighing 17–22 g (6–8 wks old, Laboratory Animal Center of the northern campus of Sun Yat-sen University, Guangzhou, China) were housed in cages with wood shavings under laboratory conditions (18°C–25°C temperature, 60%–70% humidity, and natural light). All mice had free access to food and water at the animal laboratory center of our hospital.

***In vivo* colon carcinoma model.** Donor CRC tumor cells were created by subcutaneous injection of CT26 cells (4×10^5 cells in 0.4 mL) into the nucha in BALB/c mice. Donor cells were harvested at d 10, when the tumor reached 1 cm in diameter. Orthotopic transplantation with fresh tumor tissues on herniated cecum was carried out in anesthetized BALB/c mice. A 2-cm skin incision in the left-lower quadrant was made, the skin and subcutaneous tissue were separated, and fresh donor tumor tissue cells (4×10^5 cells in 0.4 mL) were transplanted on the herniated cecum. Following suture closure, all of the mice were divided randomly into treatment groups.

In one protocol, the mice were divided into three treatment groups: normal saline, 5% DMSO, or ISO-1 (20 mg/kg in 5% DMSO). These compounds were administered twice weekly (0.2 mL by intraperitoneal injection [i.p.]) starting 3 d following transplantation. In another protocol, the mice were divided into two treatment groups: isotype-matched anti-IgG antibodies (4 mg/kg) or anti-MIF antibodies (4 mg/mL). This dose was chosen based on the successful antibody-mediated neutralization of MIF activity in other preclinical disease models (21). These antibodies were administered every other d (0.2 mL, i.p.), starting on d 2 following transplantation. Blood samples were collected by heart puncture, and mice were sacrificed 4 wks later. The tumor *in situ* and the liver were observed and measured. The whole liver was fixed in formalin, then embedded in paraffin, and made into serial slices to observe the hepatic metastases foci.

Statistical Methods

Data obtained from the study were expressed as the mean ± SEM. Statistical analysis was performed using SPSS software (version 13.0, SPSS, Chicago, IL, USA). Independent sample t test and the Mann-Whitney U test were used to determine the differences in MIF expression in different tissues and serum MIF concentration between different groups. One-way ANOVA was used to determine the MIF expression difference in colorectal tissues and serum MIF concentration among patients with different pathological alterations. Bivariate correlation was used to assess the association between colorectal epithelial MIF expression and serum MIF concentration. Multivariate analysis was performed by using the logistic regression model to evaluate various risk factors of hepatic metastasis. The numeric variables in the invasion assay and RT-PCR were determined by independent-sample *t* test. Fisher exact probabilities in a 2 × 2 table were used to determine the differences between different groups for incidence rate of hepatic metastases. Independent-sample *t* test was used to determine the differences between MIF and MMP-9 expression in serum and the weight of the tumor *in situ* and the liver between different groups. All analyses were two tailed, and the α level of significance was set at $P < 0.05$. Means, standard error of the mean (SEM), and *P* values were reported.

RESULTS

Increased Serum MIF Concentrations Correlated with Colorectal Cancer Severity in Human Patients

The demographic characteristics of the colorectal cancer patients and control group were matched for ethnic background, age, and sex. Because serum MIF protein levels correlate with disease state in a variety of cancers (22,23), we first examined MIF expression in the serum of individuals with and without colorectal cancer. MIF levels were lowest in the serum from healthy volunteers with normal colorectal mucosa and increased progressively in patients with colorectal adenoma ($P < 0.05$) and those with colorectal carcinoma ($P < 0.001$) (Figure 1). Moreover, serum MIF concentrations also correlated with the extent of metastasis. For example, among the colorectal carcinoma patients, those with either hepatic ($P = 0.03$) or lymphoid ($P = 0.03$) metastasis had significantly elevated serum

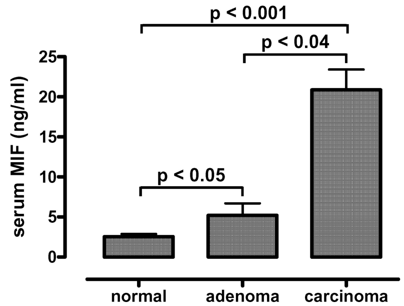

Figure 1. Human serum MIF concentrations increase progressively with colorectal cancer severity. Serum MIF concentration was low in healthy volunteers with normal colorectal mucosa, intermediate in patients with colorectal adenoma, and high in patients with colorectal carcinoma. The histograms represent the mean ± SEM; *P* values between the various groups are listed.

MIF levels relative to those patients with no metastasis. In a related finding, logistic regression analysis showed that higher serum MIF levels were associated with elevated risk of hepatic metastasis ($R = 1.25$, 95% confidence interval = 1.02–1.52, $P = 0.03$).

MIF Expression Was Increased in Cancerous Colorectal Mucosa and Metastatic Hepatic Tissue

Having shown that serum MIF levels are increased in patients with colorectal cancer, we next sought to correlate the patient's cancer severity with MIF expression within colorectal tissue. For these purposes, we scored the MIF staining on a scale of mild to intense (1 to 3) in 8 healthy volunteers, 20 age-matched patients with colorectal adenomas, and 49 age-matched patients with colorectal carcinomas (Table 1). Statistically significant associations were observed between MIF expression and tumor differentiation, lymph node status, and hepatic metastasis (Table 1; $P <$ 0.02, 0.02, and 0.03, respectively). MIF staining also showed a trend for higher intensity in the colorectal mucosa of patients with hepatic metastasis than those without hepatic metastasis (Table 1; average intensity of 2.0 ± 0.18 versus 1.5 ± 0.11, respectively). Thus, both MIF expression and MIF intensity in colorectal mucosa correlated with hepatic metastasis.

Representative colorectal tissue stained with anti-MIF antibodies is shown in Figure 2. In all cases, MIF protein was detected primarily in the cytoplasm. In agreement with the staining intensity listed in Table 1, normal colorectal mucosa displayed minimal MIF expression while colorectal adenoma and colorectal carcinoma tissue, stained in parallel, displayed progressively increased MIF expression (see Figure 2A–C). Significantly, mucosal tissue taken from patients with colorectal carcinomas that displayed normal morphology (taken at least 5 cm away from the primary tumor site) had minimal MIF staining (data not

Table 1. Increased MIF expression correlates with tumor differentiation, regional lymph node status, and liver metastasis.

MIF staining score[a]	1	2	3	*P* value
Sex				
Male	13	11	3	
Female	8	9	5	0.498
Stage				
I	4	2	1	
II	4	4	1	
III	3	4	3	
IV	10	10	3	0.866
Differentiation				
Well	8	1	0	
Moderate	10	14	4	
Poor	3	5	4	0.018
Regional lymph nodes				
N0	6	9	7	
Nx	15	11	1	
N1	5	15	0	
N2	1	8	3	
N3	0	0	4	0.017
Liver metastasis				
Yes	7	8	7	
No	14	12	1	0.027
Morphology				
Polypoid exophytic	5	2	4	
Diffuse infiltrative	3	6	0	
Ulcerative	7	9	4	
Fungating	6	3	0	0.140

[a]MIF staining was scored on a scale of 1 (weakly positive) to 3 (strongly positive).

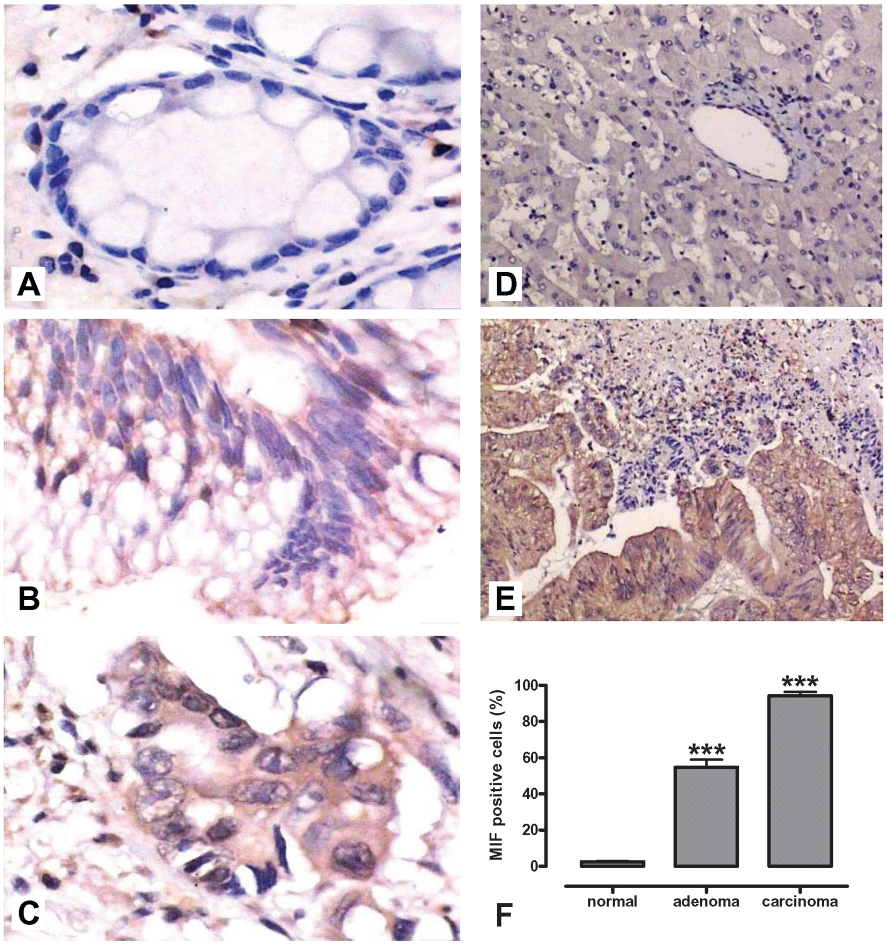

Figure 2. MIF protein expression within either primary colorectal or metastatic hepatic tissue correlates with colorectal cancer severity. Immunostaining with anti-MIF antibodies in either primary colorectal tissue: normal intestinal mucosa (A), adenoma (B), carcinoma (C), or secondary hepatic tissue: morphologically normal (D, > 5 cm from the secondary tumor site), or metastatic (E). Note the increased MIF expression, as indicated by increased brown color, in either the primary tumor—where colorectal carcinoma > colorectal adenoma > normal mucosa, or metastatic hepatic tissue. Original magnification, 400× (A–C) or 200× (D–E). The percentage of cells expressing MIF increases with colorectal cancer severity (F). Colorectal tissue from healthy volunteers or patients with adenoma or carcinoma was stained with anti-MIF antibodies and random fields (500 cells each) were scored as MIF positive. The histograms represent the mean ± SEM percentage; ***$P < 0.001$ between all groups.

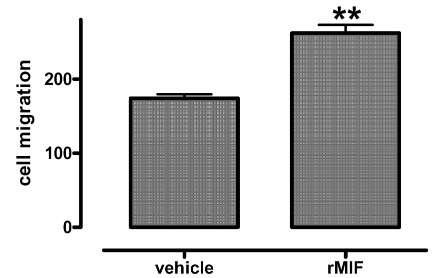

Figure 3. Recombinant MIF (rMIF) treatment increases LoVo colorectal cellular invasion. The number of cells that migrated through a transwell membrane was significantly increased by rMIF treatment (250 ng/mL, 24 h). Histograms represent the mean number of cells ± SEM measured from triplicate chambers at 24 h; **$P = 0.002$ between groups.

shown). Finally, in hepatic metastatic tissue, MIF was highly expressed (see Figure 2E) relative to that seen in hepatic tissue with normal morphology (taken at least 5 cm away from the primary tumor, see Figure 2D).

To quantitate these observations, we calculated the percentage of MIF-positive cells present in colorectal tissue by counting, in a blinded fashion, a minimum of 500 cells in each group and scoring them as either positive or negative MIF staining (see Figure 2F). The fraction of MIF-positive cells in normal mucosa (2.5%), colorectal adenoma (55%), and colorectal carcinoma (94%) increased in a progressive manner, with a significant difference between each group (see Figure 2F; $P < 0.001$). Moreover, mucosal tissue with normal morphology (taken at least 5 cm away from the primary tumor) had a low percentage of MIF staining (1.6%).

Recombinant MIF Induces Cell Invasion *in vitro*

Taken together, the results presented so far demonstrate a strong correlation between MIF expression, and both the presence of colorectal cancer and the extent of metastasis in humans. To understand whether MIF might directly contribute to cellular invasion, we assessed the invasive properties of LoVo human colon carcinoma cells in the presence and absence of recombinant MIF (rMIF) in a transwell system. In the absence of rMIF, few LoVo cells migrated through the 8-μm membrane in 24 h. In contrast, a significant number of rMIF-treated LoVo cells migrated through the membrane (Figure 3, $P = 0.002$).

Recombinant MIF Induced VEGF and a Metalloproteinase.

Utilizing the same LoVo cell culture system, we next analyzed the expression of several genes known to be upregulated in cancerous tissue. As shown in Figure 4, mRNA expression of two isoforms of VEGF (VEGF165 and VEGF121), as well as one isoform of MMP (MMP-9), increased 1.6-fold to 2.4–fold in rMIF-

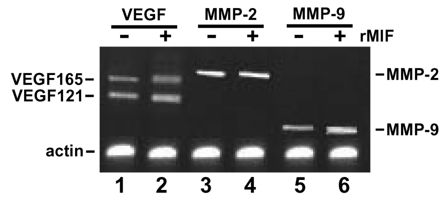

Figure 4. Recombinant MIF (rMIF) treatment increases the mRNA expression of several genes known to be upregulated in cancerous tissue. In particular, two isoforms of VEGF (165 and 121; lanes 1 and 2) and metalloproteinase-9 (lanes 5 and 6), but not metalloproteinase-2 (lanes 3 and 4), were upregulated in LoVo colorectal cells treated with rMIF (250 ng/mL, 24 h; even-numbered lanes) relative to parallel vehicle treatment (odd-numbered lanes).

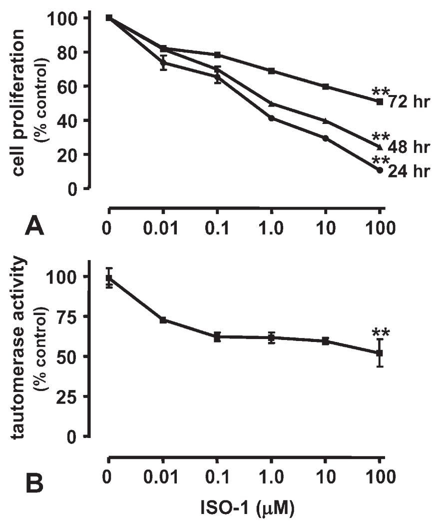

Figure 5. MIF-specific inhibition reduces colon carcinoma cell proliferation and MIF-dependent catalytic activity. (A) The MIF-specific inhibitor, ISO-1, inhibits CT26 colorectal cell proliferation. Cells were cultured under standard conditions with the inclusion of increasing concentrations of ISO-1 for either 24, 48, or 72 h, as indicated. (B) ISO-1 dose-dependently inhibits the bioactivity of MIF in cell lysates as determined by *in vitro* tautomerase activity. Plots have been normalized to the percentage of vehicle-treated control levels, ± SEM; $^{**}P < 0.01$.

treated LoVo cells relative to expression in control LoVo cells treated with PBS in parallel. In contrast, no significant difference of MMP-2 mRNA expression was observed between the rMIF-treated and control-treated LoVo cells (see Figure 4).

The MIF-Specific Inhibitor ISO-1 Reduced Colon Carcinoma Cell Proliferation and Tautomerase Activity

We have shown that MIF expression in colorectal tissue increases with cancer severity. To assess whether this intracellular MIF is biologically active, we used ISO-1, a commercially available and well-characterized nontoxic inhibitor of MIF function (19,20,21,24–27). Bcause MIF suppresses the action of the tumor suppressor p53, we reasoned that incubation of colon cancer cells with ISO-1 may decrease cancer cell proliferation by increasing p53-mediated apoptosis. In one set of MIF bioassays, therefore, we incubated murine CT26 colon carcinoma cells in the presence of increasing concentrations of ISO-1 and measured cell proliferation (Figure 5A). In a dosage- and time-dependent manner, ISO-1 inhibited both LoVo (data not shown) and CT26 cell proliferation, suggesting that increased MIF expression in cancer may inhibit p53-mediated apoptosis (see Figure 5A).

As an independent means to assess MIF bioactivity, we analyzed the tautomerase activity present in the lysates of CT26 cells treated with various concentrations of ISO-1. This assay relies on the observation that, unique among cytokines, MIF has structural homology with bacterial isomerases and tautomerizes the nonphysiological D-isomer of the tyrosine catabolite dopachrome. Although this catalytic activity is unlikely to be a natural function of MIF, *in vitro* tautomerase activity has proven to be a useful surrogate measure of MIF biological activity, because MIF's catalytic activity correlates with its proinflammatory and tumorigenic biological activities. MIF-associated tautomerase activity in cell lysates was reduced in a dose-dependent manner by increasing concentrations of the MIF inhibitor ISO-1 (see Figure 5B). Nearly identical tautomerase inhibition was observed following ISO-1 treatment of either LoVo or SW116 colorectal cells (data not shown).

MIF Inhibition Suppressed Tumor Growth *in vivo*

If MIF contributes directly to colorectal carcinogenesis, its neutralization should inhibit tumor growth. To test this prediction, we used a murine colorectal model. First, a colorectal tumor mass was propagated by subcutaneous injection of CT26 cells on the nucha of Balb/c mice. Using these tumor cells as donors, we next transplanted tumor cells on herniated caeca of 45 recipient Balb/c mice. These 45 transplant-receiving animals were divided randomly into three groups of 15; one group was treated with ISO-1 (20 mg/kg, 2 times weekly, i.p.), one with vehicle (5% DMSO, 2 times weekly, i.p.), and one with saline (2 times weekly, i.p.). Following 4 wks of treatment, the tautomerase activity of serum MIF was significantly reduced in the ISO-1–treated animals relative to that detected in either the saline-treated or vehicle-treated animals (Figure 6A, $P < 0.001$). Despite this significant reduction in serum MIF bioactivity in the ISO-1–treated animals, we observed only a non–statistically significant trend toward fewer MIF-positive cells in the colorectal tissue of the ISO-1–treated group relative to either DMSO or saline treatment (74% versus 77% and 81%, respectively; data not shown). Similarly, the body weights of the mice within each treatment group did not diverge throughout the 4-wk experiment (data not shown). The estimated tumor volume and final tumor weight of both the DMSO-treated and saline-treated control groups also remained similar throughout the experiment (data not shown). In contrast, the ISO-1–treated animals displayed significantly smaller tumor volumes and final tumor weight relative to either the DMSO-treated or saline-treated groups (see Figure 6B, C). Dissection of the cecum at wk 4 revealed a striking trend: the tumor tissues in both the DMSO- and saline-treated groups were abundant in blood supply and invasive of the neighboring organs.

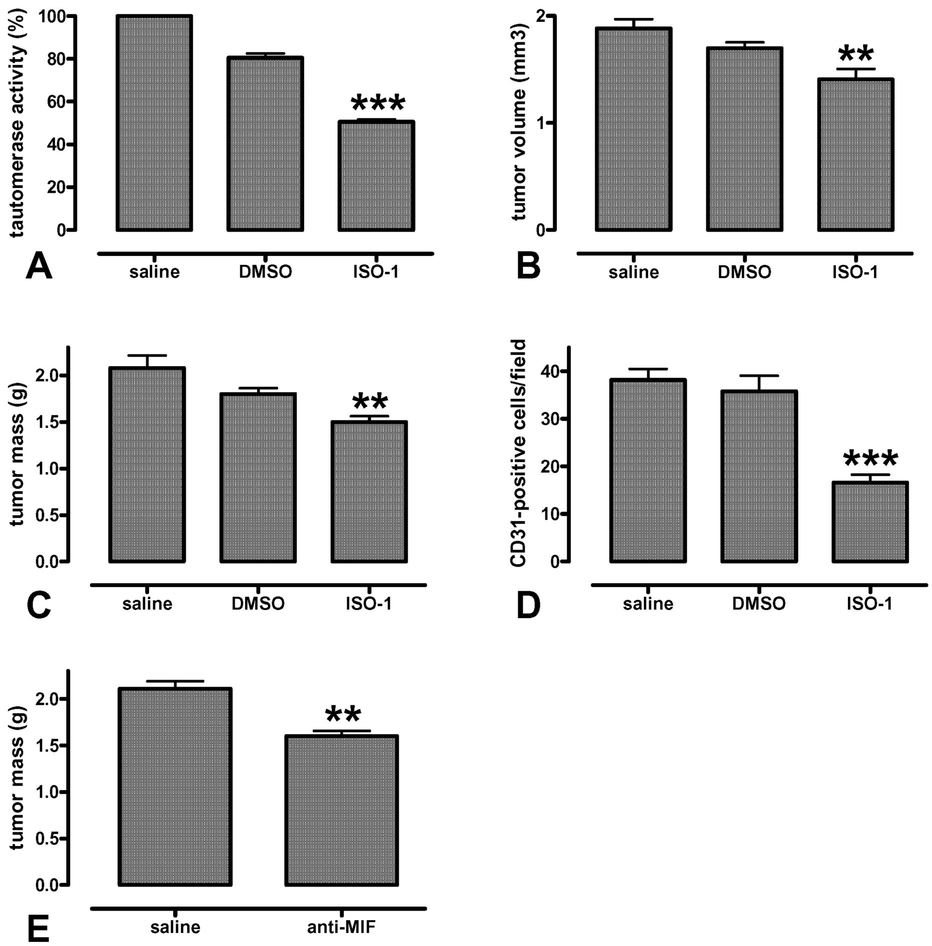

Figure 6. MIF-specific inhibition reduces colon cancer *in vivo*. (A) ISO-1 inhibits the bioactivity of serum MIF in mice. Following transplantation of CT26 tumor cells, mice were divided into three groups and treated twice weekly with saline, vehicle (5% DMSO), or the MIF-specific inhibitor ISO-1 (20 mg/kg in 5% DMSO) intraperitoneally. As a surrogate measure of MIF inhibition, the serum was collected and analyzed for MIF bioactivity as determined by *in vitro* tautomerase activity at d 28. (B and C) ISO-1 inhibits colorectal tumor burden as assessed by measuring either the tumor volume (B) or the tumor weight at d 28 of treatment (C). (D) ISO-1 inhibits angiogenesis as assessed by quantitating the number of microvessels by CD31 immunostaining. (E) Neutralizing anti-MIF antibodies inhibit colorectal tumor burden as assessed by measuring the tumor weight at d 28. Mice were given transplants of CT26 tumor cells as above and divided into two groups. Treatment consisted of either anti-MIF antibodies or isotype-matched control IgG, administered every other day, intraperitoneally. Histograms represent the mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ between anti-MIF therapy and all control groups.

In contrast, the tumors of the ISO-1–treated animals were relatively deficient in blood supply and easily separated from the neighboring organs. To characterize further the effect of ISO-1 on angiogenesis, we stained tumor tissue for CD31 (also know as platelet endothelial cell adhesion molecule PECAM-1) expression.

Consistent with the relative dearth of blood supply noted above, we observed significantly less CD31 expression in the ISO-1–treated tumor tissue (see Figure 6D). Finally, we observed one metastatic nodus on the surface of the liver of one of the saline-treated animals. All other livers, independent of treatment group, displayed normal gross morphology with no differences in size, texture, or shape. A histological examination of liver serial sections, however, revealed that the incidence of hepatic metastases in the ISO-1–treated group was significantly lower than that observed in either the DMSO- or saline-treated groups (10% versus 60% and 70%, respectively).

As an independent means to inhibit MIF activity, we utilized a neutralizing anti-MIF antibody in the same CT26-transplanted Balb/c mouse model. Briefly, 20 animals given transplanted tissue were divided into two groups: one group was treated with anti-MIF antibodies and the other with isotype-matched control antibodies. Following 4 wks of treatment, we found that the tumor in the group treated with anti-MIF antibodies was significantly reduced compared with the control group (see Figure 6E, $P < 0.01$). Additionally, the concentrations of both MIF and MMP-9 in serum of anti-MIF–treated animals were lower than that of the control group (31.15 ± 1.59 pg/mL versus 35.65 ± 1.34 pg/mL, $P < 0.01$; 0.22 ± 0.01 ng/mL versus 0.28 ± 0.04 ng/mL, $P < 0.01$). Moreover, while the weight of the liver was not significantly different between groups (0.93 ± 0.07 g versus 0.96 ± 0.07 g), the incidence of hepatic metastases in the anti-MIF–treated group was much less than that of the control-treated group (10% versus 70%, $P < 0.05$).

DISCUSSION

MIF is a multifunctional cytokine whose dysregulation plays a pivotal role in a wide variety of inflammatory and autoimmune diseases (28,29). Recently, MIF has also been implicated in a variety of cancers where it may participate in carcinogenesis through inactivating p53 (11), enhancing angiogenesis (30,31), or through Rho-dependent pathways (15). In particular, MIF has been implicated in the early stages of colorectal carcinogensis and it has been observed to be highly expressed in the gastrointestinal tract and sporadic human colorectal adenomas. MIF also facilitates tumorigenesis in the *adenomatous polyposis colimouse* model of intestinal tumorigenesis, and genetic deletion of MIF resulted in reduced tumor microvessel density (32).

In the present study, we observed significant MIF induction in both the serum and tumor specimens of patients with colorectal cancer relative to that seen in healthy volunteers. In addition, we demonstrated that increased MIF expression was correlated with an increase in both tumor differentiation and the extent of metastases (lymph node and liver), suggesting that MIF may play a crucial role in colorectal carcinogenesis and metastasis. Although the precise mode of action remains unclear, our findings that significant higher serum levels of MIF were associated with a higher risk of hepatic metastasis suggest that serum MIF levels might be a useful marker in clinical diagnosis of hepatic metastasis in colorectal carcinoma.

To better understand MIF's role in colorectal oncogenesis, we assessed whether exogenously added rMIF would increase cell invasion in the LoVo colorectal carcinoma cell line. Our results revealed that rMIF treatment significantly increased the number of LoVo cells that went through the membrane compared with the number of LoVo cells given control treatment. These observations demonstrated that rMIF enhances colorectal cell invasion *in vitro*. Similar observations have been observed in nasopharyngeal carcinoma, in which MIF enhances neoplastic cell invasion through MMP-9 and interleukin-8 induction (17). Moreover, decreasing endogenous MIF by use of siRNA dramatically inhibited CT26 cell invasion *in vitro* and liver metastasis *in vivo*, possibly through the Rho-dependent pathway (10). Taken together, these findings suggest that increased MIF expression may increase the invasion and metastasis of colorectal carcinoma.

Tumor invasion and metastasis is a complex cascade involving multiple host-tumor interactions. Essential steps include the degradation of extracellular matrix (ECM) and basement membrane (BM). MMPs belong to a gene family of zinc-containing endopeptidases, which could degrade ECM and BM to play an essential role in the metastatic process. Among members of the MMP family, MMP-2 (72-kDa type IV collagenase/gelatinase A) and MMP-9 (92-kDa type IV collagenase/gelatinase B) selectively degrade type-IV collagen, a major component of ECM and BM, and thus are significantly associated with the invasion and metastasis of tumor cells. Many studies have shown the associations of MMP-2 and/or MMP-9 with invasion and metastasis in a variety of cancers, such as nasopharyngeal carcinoma (17), and head and neck carcinomas (33). Similarly, in CRC high expression of MMP-2 and MMP-9 may be important in tumor initiation (34,35), development (36), progression, and metastasis (37). Thus, high expression of MMP-2 and MMP-9 may be used to characterize the behaviors of colorectal carcinoma (38,39). Moreover, high colorectal MMP-9 expression has been implicated in lung metastases (40) and tumor cell resistance to proapoptotic and p53 effects, as well as poor efficacy in postoperative adjuvant chemotherapy (41). *In vitro*, MIF also was shown to promote cell invasiveness through macrophage secretion of MMP (42). Herein, we extend this MMP-9 CRC correlation by demonstrating a strong positive correlation between MMP-9 expression and the extent of both primary colorectal cancer and its extent of metastases.

Unlike the MMP-9 induction discussed above, MMP-2 expression was not increased by rMIF treatment in our hands. Our results confirmed the findings from a previous study (43), which demonstrated that recombinant rat MIF upregulated MMP-9 and MMP-13 mRNA dramatically, but did not significantly alter MMP-2 mRNA expression.

The formation of new blood vessels is essential for tumor growth and is controlled by angiogenic factors that are secreted primarily by tumor cells (16). Among the angiogenic factors, VEGF is considered a very important growth factor in neovascularization cancer progression (44). VEGF induction is associated with increases in microvascular density, which in turn is directly correlated with increased metastasis of many cancers, including colorectal cancer (45). For example, colonic VEGF induction is correlated with tumor angiogenesis (46), tumor progression (47), and metastatic potential, particularly to the liver in individuals with colorectal cancers (48). Similarly, serum VEGF levels are higher in patients with advanced colorectal cancers relative to healthy volunteers (49–52). Finally, anti-VEGF therapy inhibits hepatic metastasis from colorectal cancer (53). In agreement with this work, our studies in LoVo cells show that rMIF induced VEGF. We postulate that MIF may be involved in the regulation of antigenic factors, such as increasing the secretion of VEGF to promote tumorigenesis. Interestingly, we and others observed a strong correlation between MMP-9 and VEGF levels, suggesting they may share a common induction pathway (54).

CONCLUSION

Our results demonstrate that MIF is more highly expressed in colorectal adenoma and carcinoma tissues, and is also elevated in the sera of patients with colorectal cancer. Moreover, MIF upregulation is associated with increased tumor differentiation, lymph node invasion, and hepatic metastasis, consistent with the intimate involvement of MIF in colorectal carcinogenesis and metastasis. Our most compelling evidence implicating MIF in colorectal cancer comes from an *in vivo* model in which mice carrying colon carcinoma transplants have a significantly reduced tumor burden when treated with either the MIF-specific small molecule inhibitor ISO-1 or with neutralizing anti-MIF antibodies. Finally, our *in vitro* findings suggest that MIF may enhance colorectal tumor growth and invasion through induction of VEGF and MMP-9. These findings suggest that the level of MIF in both serum and colorectal tissue may be a useful marker in the diagnosis of colorectal cancer and its metastasis to the liver.

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DISCLOSURE

We declare that the authors have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper. YA Al-Abed is the inventor of patents covering ISO-1 and analogs thereof, which have been licensed for further development.

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